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10

### Modified cDNA Factor VIII and its Derivatives

15 The present invention relates to modified DNA sequences coding for biologically  
active recombinant human factor VIII and its derivatives with improved stability,  
recombinant expression vectors containing such DNA sequences, host cells  
transformed with such recombinant expression vectors, and processes for the  
manufacture of the recombinant human factor VIII and its derivatives. The invention  
20 also covers a transfer vector for use in human gene therapy which comprises such  
modified DNA sequences.

Classic hemophilia or hemophilia A is the most common of the inherited bleeding  
disorders. It results from a chromosome X-linked deficiency of blood coagulation  
25 factor VIII, and affects almost exclusively males with an incidence of between one  
and two individuals per 10.000. The X-chromosome defect is transmitted by female  
carriers who are not themselves hemophiliacs. The clinical manifestation of  
hemophilia A is an abnormal bleeding tendency and before treatment with factor  
VIII concentrates was introduced the mean life span for a person with severe  
30 hemophilia was less than 20 years. The use of concentrates of factor VIII from  
plasma has considerably improved the situation for the hemophilia patients. The  
mean life span has increased extensively, giving most of them the possibility to live  
a more or less normal life. However, there have been certain problems with the  
plasma derived concentrates and their use, the most serious of which have been  
35 the transmission of viruses. So far, viruses causing AIDS, hepatitis B, and non A

5 non B hepatitis have hit the population seriously. Although different virus inactivation methods and new highly purified factor VIII concentrates have recently been developed an inadvertant contamination can not be excluded. Also, the factor VIII concentrates are fairly expensive because of the limited supply of human plasma raw material.

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A factor VIII product derived from recombinant material is likely to solve a large extent of the problems associated with the use of plasma derived factor VIII concentrates for treatment for hemophilia A. However, the development of a recombinant factor VIII has met some difficulties, for instance the problem of  
15 achieving production levels in sufficiently high yields, in particular regarding the full-length molecule.

In fresh plasma prepared in the presence of protease inhibitors, factor VIII has been shown to have a molecular weight of 280 kDa and to be composed of two  
20 polypeptide chains of 200 kDa and 80 kDa, respectively (Andersson, L.-O., et al. (1986) Proc. Natl. Acad. Sci. USA 83, 2979-2983). These chains are held together by metal ion bridges. More or less proteolytically degraded forms of the factor VIII molecule can be found as active fragments in factor VIII material purified from commercial concentrates (Andersson, L.-O., et al. ibid.; Andersson, L.-O., et al.  
25 (1985) EP 0 197 901). The fragmented form of factor VIII having molecular weights from 260 kDa down to 170 kDa, consists of one heavy chain with a molecular weight ranging from 180 kDa down to 90 kDa, where all variants have identical amino termini, in combination with one 80 kDa light chain. The amino-terminal region of the heavy chain is identical to that of the single chain factor VIII  
30 polypeptide that can be deduced from the nucleotide sequence data of the factor VIII cDNA (Wood, W.I., et al. (1984) Nature 312, 330-336; Vehar, G.A., et al. (1984) Nature 312, 337-342).

The smallest active form of factor VIII with a molecular weight of 170 kDa,  
35 consisting of one 90 kDa and one 80 kDa chain, can be activated with thrombin to

5 the same extent as the higher molecular weight forms, and thus represents an unactivated form. It has also been shown to have full biological activity in vivo as tested in hemophilia dogs (Brinkhous, K.M., et al. (1985) Proc. Natl. Acad. Sci. USA 82, 8752-8756). Thus, the haemostatic effectiveness of the 170 kDa form is the same as for the high molecular weight forms of factor VIII.

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The fact that the middle heavily glycosylated region of the factor VIII polypeptide chain residing between amino acids Arg-740 and Glu-1649 does not seem to be necessary for full biological activity has prompted several researchers to attempt to produce derivatives of recombinant factor VIII lacking this region. This has been  
15 achieved by deleting a portion of the cDNA encoding the middle heavily glycosylated region of factor VIII either entirely or partially.

For example, J.J. Toole, et al, reported the construction and expression of factor VIII lacking amino acids 982 through 1562, and 760 through 1639 respectively  
20 (Proc. Natl. Acad. Sci. USA (1986) 83, 5939-5942). D.L. Eaton, et al. reported the construction and expression of factor VIII lacking amino acids 797 through 1562 (Biochemistry (1986) 25, 8343-8347). R.J. Kaufman described the expression of factor VIII lacking amino acids 741 through 1646 (PCT application No. WO 87/04187). N. Sarver, et al. reported the construction and expression of factor VIII  
25 lacking amino acids 747 through 1560 (DNA (1987) 6, 553-564). M. Pasek reported the construction and expression of factor VIII lacking amino acids 745 through 1562, and amino acids 741 through 1648, respectively (PCT application No. WO 88/00831). K.-D. Langner reported the construction and expression of factor VIII lacking amino acids 816 through 1598, and amino acids 741 through 1689,  
30 respectively (Behring Inst. Mitt., (1988) No. 82, 16-25, EP 0 295 597). P. Meulien, et al., reported the construction and expression of factor VIII lacking amino acids 868 through 1562, and amino acids 771 through 1666, respectively (Protein Engineering (1988) 2(4), 301-306, EP 0 303 540). When expressing these deleted forms of factor VIII cDNA in mammalian cells the production level is typically 10 times higher  
35 as compared to full-length factor VIII.

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FVIII is secreted into plasma as a heterodimer of a heavy chain (domains A1-A2-B) and a light chain (A3-C1-C2) associated through a noncovalent divalent metal ion linkage between the A1- and A3 domains. In plasma, FVIII is stabilized by binding to von Willebrand factor.

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Upon proteolytic activation by thrombin, FVIII is activated to a heterotrimer of 2 heavy chain fragments (A1, a 50 kDa fragment, and A2 a 43 kDa fragment) and the light chain (A3-C1-C2, a 73 kDa fragment). The active form of FVIII (FVIIIa) thus consists of an A1-subunit associated through the divalent metal ion linkage to a thrombin-cleaved A3-C1-C2 light chain and a free A2 subunit associated with the A1 domain. The dissociation of that free A2 subunit from the heterotrimer is thought to be the rate limiting step in FVIIIa inactivation after thrombin activation (Fay, P.J. et al, J. Biol. Chem. 266: 8957 (1991), Fay PJ & Smudzin TM, J. Biol. Chem. 267: 13246-50 (1992)). The half life of FVIIIa in plasma is only 2.1 minutes (Saenko et al., Vox Sang. 83: 89-96 (2002)). To enhance the half life of FVIIIa would result into a longer acting FVIIIa which would also translate into less frequent injections of such a FVIII preparation. The inactivation of FVIIIa through activated Protein C (APC) by cleavage at Arg336 and Arg562 is thought not to be the rate limiting step. Attempts have been made to create a FVIIIa which is inactivation resistant by covalently attaching the A2 domain to the A3 domain and by mutating the APC cleavage sites (Pipe and Kaufman, PNAS; 94:11851-11856). However such a FVIIIa could have a thrombogenic potential as it is almost completely inactivation resistant. It is therefore the purpose of this invention to create a FVIIIa in which the A2 domain is stabilized without completely blocking inactivation.

30

FVIII is administered i.v. to haemophilia patients who are on prophylactic treatment about 3 times per week due to the plasma half life of FVIII of about 12 hours. It would thus be highly desirable to create a FVIII with enhanced plasma half life which could lead to a FVIII preparation which has to be administered less

35

5 frequently. The present invention offers a solution to this problem by a modified FVIII molecule with an increased association of A2 to the A1/A3-C1-C2.

The nature of these modifications was identified by comparing the sequence of porcine FVIII to that of human FVIII as it is known that the dissociation of human A2 domain is threefold enhanced versus that of porcine A2 (Lollar et al., J Biol. Chem., 10 267:23652-23657 (1992)). The sequence comparison (Fig. 1) revealed several differences. A subset of these differences consists of differently charged amino acids. Mutants of human FVIII were constructed according to the following guidelines. When the human sequence contained a neutral amino acid whereas the 15 porcine sequence contained a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence was introduced into the human sequence. When the human sequence contained a charged amino acid whereas the porcine FVIII contained a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge was introduced, e.g. if the human FVIII contained 20 an acidic amino acid at a position where the porcine FVIII contained a neutral amino acid, also a basic amino acid was introduced. When the human sequence contained a charged sequence whereas the porcine FVIII contained a charged amino acid then an amino acid with the same charge as found in the porcine amino acid was introduced into the human sequence. Examples for such mutations which 25 lead to an improved FVIII with a plasma half life of its activated form of more than three minutes, preferably of more than 10 minutes even more preferably more than 30 minutes, are listed in figure 2.

Other mutations for an improved FVIII were deduced by analyzing mutations in 30 human FVIII which occurred naturally and which lead to a faster dissociation of the A2 domain associated with hemophilia. Such mutations result in differences between the two-stage assay as compared to the one-stage assay while determining FVIII clotting activity, whereas the two-stage assay result is lower than that of the one-stage assay as in the two-stage assay an incubation time of several 35 minutes allows an unstable A2 domain to dissociate (Saenko et al., Vox Sang.,

5 83:89-96 (2002). It was inferred that in those cases where such an increased instability was the result of the introduction of a charged amino acid that amino acid should be mutated into one of the opposite charge. Examples for such mutations which lead to an improved FVIII with a plasma half life of its activated form of more than three minutes, preferably of more than 10 minutes, even more preferably more than 30 minutes, are listed in figure 3.

As a basis for introducing the mutations preferably a modified factor VIII cDNA is used which comprises a first DNA segment coding for the amino acids 1 through 740 of the human factor VIII and a second DNA segment coding for the amino acids 15 1649 through 2332 of the human factor VIII. These two segments may be interconnected by a linker DNA segment, but the invention also encompasses introducing the mutations into full length FVIII.

Subject of the invention is therefore a modified human factor VIII cDNA wherein mutations are inserted either in the wild-type factor VIII cDNA or in a factor VIII 20 cDNA in which the B-domain is partially or completely deleted and may be replaced by a DNA linker segment, and

A) one or several codons of the human factor VIII cDNA which are not identical with the corresponding codon in the same position of the porcine factor VIII cDNA 25 are substituted by a different codon in such a way that

- when the human sequence contains a codon for a neutral amino acid whereas the porcine sequence contains a codon for a charged amino acid then a codon for an amino acid with the same charge as found in 30 the porcine sequence is introduced into the human sequence;
- when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for a neutral amino acid then a codon for a neutral amino acid or a codon for an amino acid of the 35 opposite charge is introduced into the human sequence

5

- when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for an amino acid with the opposite charge then a codon for an amino acid with the opposite charge is introduced into the human sequence or

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B) one or several codons for a charged amino acid which are found in the FVIII cDNA of a hemophilic patient are replaced by a codon for an amino acid of the opposite charge.

15 The production of factor VIII proteins at high levels in suitable host cells, requires the assembly of the above-mentioned modified factor VIII DNA's into efficient transcriptional units together with suitable regulatory elements in a recombinant expression vector, that can be propagated in E. coli according to methods known to those skilled in the art. Efficient transcriptional regulatory elements could be derived  
20 from viruses having animal cells as their natural hosts or from the chromosomal DNA of animal cells. Preferably, promoter-enhancer combinations derived from the Simian Virus 40, adenovirus, BK polyoma virus, human cytomegalovirus, or the long terminal repeat of Rous sarcoma virus, or promoter-enhancer combinations including strongly constitutively transcribed genes in animal cells like beta-actin or  
25 GRP78 can be used. In order to achieve stable high levels of mRNA transcribed from the factor VIII DNA's, the transcriptional unit should contain in its 3'-proximal part a DNA region encoding a transcriptional termination-polyadenylation sequence. Preferably, this sequence is derived from the Simian Virus 40 early transcriptional region, the rabbit beta-globin gene, or the human tissue plasminogen activator  
30 gene.

The factor VIII cDNA's are then integrated into the genome into a suitable host cell line for expression of the factor VIII proteins. Preferably this cell line should be an animal cell-line of vertebrate origin in order to ensure correct folding, disulfide bond  
35 formation, asparagines-linked glycosylation and other post-translational

5 modifications as well as secretion into the cultivation medium. Examples on other post-translational modifications are tyrosine O-sulfation, and proteolytic processing of the nascent polypeptide chain. Examples of cell lines that can be use are monkey COS-cells, mouse L-cells, mouse C127-cells, hamster BHK-21 cells, human embryonic kidney 293 cells, and preferentially CHO-cells.

10

The recombinant expression vector encoding the factor VIII cDNA's can be introduced into an animal cell line in several different ways. For instance, recombinant expression vectors can be created from vectors based on different animal viruses, Examples of these are vectors based on baculovirus, vaccinia virus, 15 adenovirus, and preferably bovine papilloma virus.

The transcription units encoding the factor VIII DNA's can also be introduced into animal cells together with another recombinant gene which may function as a dominant selectable marker in these cells in order to facilitate the isolation of 20 specific cell clones which have integrated the recombinant DNA into their genome. Examples of this type of dominant selectable marker genes are Tn5 aminoglycoside phosphotransferase, conferring resistance to Geneticin (G418), hygromycin phosphotransferase, conferring resistance to hygromycin, and puromycin acetyl transferase, conferring resistance to puromycin. The recombinant expression vector 25 encoding such a selectable marker can reside either on the same vector as the one encoding the factor VIII cDNA, or it can be encoded on a separate vector which is simultaneously introduced and integrated to the genome of the host cell, frequently resulting in a tight physical linkage between the different transcription units.

30 Other types of selectable marker genes which can be used together with the factor VIII DNA's are based on various transcription units encoding dihydrofolate reductase (dhfr). After introduction of this type of gene into cells lacking endogenous dhfr-activity, preferentially CHO-cells (DUKX-B11, DG-44) it will enable these to grow in media lacking nucleosides. An example of such a medium is 35 Ham's F12 without hypoxanthin, thymidin, and glycine. These dhfr-genes can be



5 introduced together with the factor VIII cDNA transcriptional units into CHO-cells of the above type, either linked on the same vector or on different vectors, thus creating dhfr-positive cell lines producing recombinant factor VIII protein.

10 If the above cell lines are grown in the presence of the cytotoxic dhfr-inhibitor methotrexate, new cell lines resistant to methotrexate will emerge. These cell lines may produce recombinant factor VIII protein at an increased rate due to the amplified number of linked dhfr and factor VIII transcriptional units. When propagating these cell lines in increasing concentrations of methotrexate (1-10000 nM), new cell lines can be obtained which produce factor VIII protein at very high  
15 rate.

The above cell lines producing factor VIII protein can be grown on a large scale, either in suspension culture or on various solid supports. Examples of these supports are microcarriers based on dextran or collagen matrices, or solid supports  
20 in the form of hollow fibres or various ceramic materials. When grown in cell suspension culture or on microcarriers the culture of the above cell lines can be performed either as a bath culture or as a perfusion culture with continuous production of conditioned medium over extended periods of time. Thus, according to the present invention, the above cell lines are well suited for the development of  
25 an industrial process for the production of recombinant factor VIII that can be isolated from human plasma.

The recombinant factor VIII protein which accumulate in the medium of CHO-cells of the above type, can be concentrated and purified by a variety of biochemical and  
30 chromatographic methods, including methods utilizing differences in size, charge, hydrophobicity, solubility, specific affinity, etc. between the recombinant factor VIII protein and other substances in the cell cultivation medium.

An example of such a purification is the adsorption of the recombinant factor VIII  
35 protein to a monoclonal antibody which is immobilised on a solid support. After

5 desorption, the factor VIII protein can be further purified by a variety of chromatographic techniques based on the above properties.

The recombinant proteins with factor VIII activity described in this invention can be formulated into pharmaceutical preparations for therapeutic use. The purified factor  
10 VIII proteins may be dissolved in conventional physiologically compatible aqueous buffer solutions to which there may be added, optionally, pharmaceutical adjuvants to provide pharmaceutical preparations.

The modified factor VIII DNA's of this invention may also be integrated into a  
15 transfer vector for use in the human gene therapy.

A further subject of this invention is a modified biologically active recombinant human factor VIII with improved plasma half life of its activated form wherein mutations are inserted either in the wild-type factor VIII or in a FVIII in which the B-  
20 domain is partially or completely deleted and replaced by a linker, and

A) one or several amino acids of the human factor VIII which are not identical with the corresponding amino acid in the same position of the porcine factor VIII are substituted by a different amino acid in such a way that

- 25
- when the human sequence contains a neutral amino acid whereas the porcine sequence contains a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;
- 30
- when the human sequence contains a charged amino acid whereas the porcine sequence contains a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge is introduced into the human sequence;

- 5                   • when the human sequence contains a charged amino acid whereas the porcine sequence contains an amino acid with the opposite charge then an amino acid with the opposite charge is introduced into the human sequence or

- 10    B)    one or several charged amino acids which are found in the FVIII amino sequence of hemophilic patients are replaced by a codon for an amino acid of the opposite charge.

The present invention will be further described more in detail in the following  
15   examples thereof. This description of specific embodiments of the invention will be made in conjunction with the appended figures.

#### **Generation of FVIII mutants**

For the generation of FVIII mutants, a suitable subfragment of the FVIII cDNA (e.g.  
20   Aval - SacI, encompassing aminoacids 226 to 978) is first subcloned into a suitable cloning vector to reduce subsequent sequencing efforts. Site directed mutagenesis is then performed with a commercially available mutagenesis kit (e.g. QuickChange SiteDirected Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Primers used for mutagenesis are listed in the attached sequence  
25   listing and below, where the mutagenic bases are indicated in bold letters.

Mutation:           A284K

Forward primer

5'GGAACCATCGCCAGAAGTCCTTGGAAATCTCGCC<sup>3'</sup>           (Sequence 1)

30   Reverse primer

5'GGCGAGATTTC<sup>3'</sup>CAAGGACTTCTGGCGATGGTTCC<sup>3'</sup>           (Sequence 2)

Mutation:       D318G

Forward primer

35   5'CCCACCAACATGGTGGCATGGAAGCTTATGTC<sup>3'</sup>           (Sequence 3)

5 Reverse primer

5'GACATAAGCTTCCATGCCACCATGTTGGTGGG<sup>3'</sup> (Sequence 4)

Mutation: M337R

Forward

primer

10 5'CAGAGGAACCCCAACTACGACGTAAAAATAATGAAGAAGCGGAAGAC<sup>3'</sup>  
(Sequence 5)

Reverse

primer

5'GTCTTCCGCTTCTTCATTATTTTTACGTCGTAGTTGGGGTTCCTCTG<sup>3'</sup>  
(Sequence 6)

15

Mutation: N340D

Forward

primer

5'CCCAACTACGAATGAAAAATGATGAAGAAGCGGAAGACTATG<sup>3'</sup>  
(Sequence 7)

20 Reverse

primer

5'CATAGTCTTCCGCTTCTTCATCATTTCATTCGTAGTTGGG<sup>3'</sup>  
(Sequence 8)

Mutation: D349N

25 Forward

primer

5'GAAGAAGCGGAAGACTATGATGATAATCTTACTGATTCTG<sup>3'</sup>  
(Sequence 9)

Reverse primer 5'CAGAATCAGTAAGATTATCATCATAGTCTTCCGCTTCTTC<sup>3'</sup>  
(Sequence 10)

30

Mutation: N364D

Forward primer 5'GGTCAGGTTTGATGATGACGACTCTCCTTCCTTTATCC<sup>3'</sup>  
(Sequence 11)

Reverse primer 5'GGATAAAGGAAGGAGAGTCGTCATCATCAAACCTGACC<sup>3'</sup>  
(Sequence 12)

35

5

Mutation: D403S

Forward primer 5'CCCTTAGTCCTCGCCCCCTCTGACAGAAGTTATAAAAG<sup>3'</sup>  
(Sequence 13)

Reverse primer 5'CTTTTATAACTTCTGTCAGAGGGGGCGAGGACTAAGGG<sup>3'</sup>  
10 (Sequence 14)

Mutation: E434V

Forward primer  
5'GTCCGATTTATGGCATAACACAGATGTTACCTTTAAGACTCG<sup>3'</sup>  
15 (Sequence 15)

Reverse primer  
5'CGAGTCTTAAAGGTAACATCTGTGTATGCCATAAATCGGAC<sup>3'</sup>  
(Sequence 16)

20

Mutation: E440K

Forward primer  
5'CCTTTAAGACTCGTAAAGCTATTCAGCATGAATCAGG<sup>3'</sup> (Sequence 17)

Reverse primer  
25 5'CCTGATTCATGCTGAATAGCTTTACGAGTCTTAAAGG<sup>3'</sup> (Sequence 18)

Mutation: Q468K

Forward primer  
5'CACACTGTTGATTATATTTAAGAATAAAGCAAGCAGACCATATAAC<sup>3'</sup>  
30 (Sequence 19)

Reverse primer  
5'GTTATATGGTCTGCTTGCTTTATTCTTAAATATAATCAACAGTGTG<sup>3'</sup>  
(Sequence 20)

35 Mutation: R484S

5 Forward primer

5'CCCTCACGGAATCACTGATGTCTCTCCTTTGTATTCAAGG<sup>3</sup>

(Sequence 21)

Reverse

primer

5'CCTTGAATACAAAGGAGAGACATCAGTGATTCCGTGAGGG<sup>3</sup>

10 (Sequence 22)

Mutation: R489G

Forward primer 5'GATGTCCGTCCTTTGTATTCAGGGAGATTACCAAAAGG<sup>3</sup>

(Sequence 23)

15 Reverse primer 5'CCTTTTGGTAATCTCCCTGAATACAAAGGACGGACATC<sup>3</sup>

(Sequence 24)

Mutation: R583Q

Forward primer

20 5'CTGTATTTGATGAGAACCAAAGCTGGTACCTCACAG<sup>3</sup> (Sequence 25)

Reverse primer

5'CTGTGAGGTACCAGCTTTGGTTCTCATCAAATACAG<sup>3</sup> (Sequence 26)

Mutation: A599D

25 Forward primer

5'CTCCCAATCCAGATGGAGTGCAGCTTGAG<sup>3</sup> (Sequence 27)

Reverse primer

5'CTCAAGCTGCACTCCATCTGGATTGGGGAG<sup>3</sup> (Sequence 28)

30 Mutation: E604Q

Forward primer

5'CAGCTGGAGTGCAGCTTCAGGATCCAGAGTTC<sup>3</sup> (Sequence 29)

Reverse primer

5'GAACTCTGGATCCTGAAGCTGCACTCCAGCTG<sup>3</sup> (Sequence 30)

35

5 Mutation: G1948K

Forward

primer

5' CGATGGTATCTGCTCAGCATGAAGAGCAATGAAAACATCCATTCTATT  
C<sup>3'</sup> (Sequence 31)

Reverse

primer

10 5' GAATAGAATGGATGTTTTTCATTGCTCTTCATGCTGAGCAGATACCATCG  
3' (Sequence 32)

After clone isolation and sequence verification mutant subfragments are reinserted into the respective expression vector.

15

#### Expression of FVIII mutants

Transfection of FVIII mutant clones and expression of the mutant FVIII molecules is done as described previously and known to those skilled in the art (e.g. Plantier JL et al. Thromb. Haemost. 86:596-603 (2001)).

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5 **Measuring affinity of A2 subunit for A1/A3-C1-C2**

The increased affinity of the A2 subunit for the A1/A3-C1-C2 can be measured as previously described by functional assays (Fay PJ & Smudzin TM. J. Biol. Chem. 267:13246-50 (1992); Lollar P et al. J. Biol. Chem. 267:23652-57 (1992)) as well as a physical assay employing surface plasmon resonance (Persson E et al. Biochemistry 34:12775-81 (1995)).

The sequence of the porcine factor VIII is shown in Sequence 33, whereas the sequence of the human factor VIII is shown in sequence 34 of the attached sequence listing.

15



241 . . . . . K . . . . . Porcine FVIII  
240 R S L P G L I G C H R K S V Y W H V I G Human FVIII

261 . . . . . S . . . . . Porcine FVIII  
260 M G T T P E V H S I F L E G H T F L V R Human FVIII

281 H . . . . . L . . . . . F Porcine FVIII  
280 N H R Q A S L E I S P I T F L T A Q T L Human FVIII

301 . . . . . H . G . . . . . Porcine FVIII  
300 L M D L G Q F L L F C H I S S H Q H D G Human FVIII

321 . . . . . H . R . E . . . . . R . A Porcine FVIII  
320 M E A Y V K V D S C P E E P Q L R M K N Human FVIII

341 D . . . . . N . Y . . . . . D Porcine FVIII  
340 N E E A E D Y D D D L T D S E M D V V R Human FVIII

360 L . G . D V S P . . . . . Porcine FVIII  
360 F D D D N S P S F I Q I R S V A K K H P Human FVIII

380 . . . . . S . . . . . A . . . . . Porcine FVIII  
380 K T W V H Y I A A E E E D W D Y A P L V Human FVIII

400 P S . S . . . . . L . . . . . S . . . . . Porcine FVIII  
400 L A P D D R S Y K S Q Y L N N G P Q R I Human FVIII

420 . . . . . A . . . . . V . . . . . V . . . . . Porcine FVIII  
420 G R K Y K K V R F M A Y T D E T F K T R Human FVIII

440 K . . . . . P Y . . . . . Porcine FVIII  
440 E A I Q H E S G I L G P L L Y G E V G D Human FVIII

460 . . . . . K . . . . . Porcine FVIII  
460 T L L I I F K N Q A S R P Y N I Y P H G Human FVIII

480 . . . . . S A . H P G . . . . . L . . . . . W . . . . . Porcine FVIII  
480 I T D V R P L Y S R R L P K G V K H L K Human FVIII

500 M . . . . . T . . . . . Porcine FVIII  
500 D F P I L P G E I F K Y K W T V T V E D Human FVIII

520 . . . . . S I . L . . . . . Porcine FVIII  
520 G P T K S D P R C L T R Y Y S S F V N M Human FVIII

540 K . . . . . Porcine FVIII  
540 E R D L A S G L I G P L L I C Y K E S V Human FVIII

560 . . . . . M . . . . . Porcine FVIII  
560 D Q R C N Q I M S D K R N V I L F S V F Human FVIII

580 . . . . . Q . . . . . A . . . . . D . . . . . Porcine FVIII  
580 D E N R S W Y L T E N I Q R F L P N P A Human FVIII

600 L . P Q . . . . . Porcine FVIII  
600 G V Q L E D P E F Q A S N I M H S I N G Human FVIII

5

~~Fig. 2: Mutations of human FVIII which are based on a protein comparison with porcine FVIII (does not comprise all mutations which are possible according to the invention);~~

<del>aa (mature human FVIII)</del>	<del>Amino acid in human sequence</del>	<del>Mutated to</del>	<del>Or mutated to</del>
318	D	G	K
337	M	R	
340	N	D	
349	D	N	K
364	N	D	
403	D	S	K
434	E	V	K
440	E	K	
468	Q	K	
484	R	S	E
489	R	G	E
583	R	Q	E
599	A	D	
604	E	Q	K

5

~~Fig. 3: Mutations of human FVIII which are based on an analysis of existing human mutations leading to an enhanced dissociation of the A2 domain (does not comprise all mutations possible according to the invention)~~

aa (Mature human FVIII)	From (human sequence)	To	Comments
284	A	K	A to E leads to enhanced dissociation of A2 domain
1948	G	K	G to D leads to enhanced dissociation of A2 domain

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20 In the following Sequence Listing Sequences 1-32 describe oligonucleotides which are used to introduce specific mutations into FVIII. Sequence 33 is the amino acid sequence of full length mature porcine FVIII, Sequence 34 is the amino acid sequence of full length mature human FVIII.

5 Sequence Listing

Organization Applicant

-----  
Street : Emil-von-Behring-Straße 76  
City : Marburg  
10 State : Hessen  
Country : Germany  
PostalCode : 35041  
PhoneNumber : 0049-6421-39 45 06  
FaxNumber : 0049-6421-39 45 58  
15 EmailAddress : Hugo.Pfeil@aventis.com  
<110> OrganizationName : Aventis Behring GmbH

Application Project

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20 <120> Title : Modified cDNA Factor VIII and its Derivatives  
<130> AppFileReference : 2002/M018 (A66)  
<140> CurrentAppNumber :  
<141> CurrentFilingDate : \_\_\_\_-\_\_\_\_-\_\_\_\_

25 Sequence

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<213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
ggaaccatcg ccagaagtc ttggaaatct cgcc

30 <212> Type : DNA

<211> Length : 34

SequenceName : Sequenz 1

SequenceDescription :

35 Feature

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Sequence: Sequenz 1:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

40 <222> LocationTo : 34

Other Information :

CDSJoin : No

5 Custom Codon  
Sequence Name : Sequenz 1

Sequence  
-----

10 <213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
ggcgagattt ccaaggactt ctggcgatgg ttcc  
<212> Type : DNA  
<211> Length : 34

34

15 SequenceName : Sequenz 2  
SequenceDescription :

Feature  
-----

20 Sequence: Sequenz 2:  
<221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
<222> LocationTo : 34  
Other Information :  
25 CDSJoin : No

Custom Codon  
-----

Sequence Name : Sequenz 2

30

Sequence  
-----

<213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
35 cccaccaaca tggatggcatg gaagcttatg tc  
<212> Type : DNA  
<211> Length : 32

32

SequenceName : Sequenz 3  
SequenceDescription :

40

Feature  
-----

5 Sequence: Sequenz 3:  
<221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
<222> LocationTo : 32  
Other Information :  
10 CDSJoin : No

Custom Codon  
-----

Sequence Name : Sequenz 3

Sequence  
-----

15 <213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
20 gacataagct tccatgccac catgttggtg gg 32  
<212> Type : DNA  
<211> Length : 32

SequenceName : Sequenz 4  
SequenceDescription :

25 Feature  
-----

Sequence: Sequenz 4:  
30 <221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
<222> LocationTo : 32  
Other Information :  
CDSJoin : No

35 Custom Codon  
-----

Sequence Name : Sequenz 4

Sequence  
-----

40 <213> OrganismName : Artificial Sequence  
<400> PreSequenceString :

5 cagaggaacc ccaactacga cgtaaaaata atgaagaagc ggaagac 47

<212> Type : DNA

<211> Length : 47

SequenceName : Sequenz 5

SequenceDescription :

10

Feature

-----

Sequence: Sequenz 5:

<221> FeatureKey : primer\_bind

15

<222> LocationFrom : 1

<222> LocationTo : 47

Other Information :

CDSJoin : No

20

Custom Codon

-----

Sequence Name : Sequenz 5

Sequence

25

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

gtcttcgcgt tcttcattat ttttacgtcg tagttggggg tctctcg 47

<212> Type : DNA

30

<211> Length : 47

SequenceName : Sequenz 6

SequenceDescription :

Feature

35

-----

Sequence: Sequenz 6:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 47

40

Other Information :

CDSJoin : No

5 Custom Codon

-----

Sequence Name : Sequenz 6

Sequence

10 -----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

cccaactacg aatgaaaaat gatgaagaag cggaagacta tg

42

<212> Type : DNA

15 <211> Length : 42

SequenceName : Sequenz 7

SequenceDescription :

Feature

20 -----

Sequence: Sequenz 7:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 42

25 Other Information :

CDSJoin : No

Custom Codon

-----

30 Sequence Name : Sequenz 7

Sequence

-----

<213> OrganismName : Artificial Sequence

35 <400> PreSequenceString :

catagtcttc cgcttcttca tcatttttca ttcgtagttg gg

42

<212> Type : DNA

<211> Length : 42

SequenceName : Sequenz 8

40 SequenceDescription :

Feature



5

-----

Sequence: Sequenz 8:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 42

10

Other Information :

CDSJoin : No

Custom Codon

-----

15

Sequence Name : Sequenz 8

Sequence

-----

<213> OrganismName : Artificial Sequence

20

<400> PreSequenceString :

gaagaagcgg aagactatga tgataatctt actgattctg

40

<212> Type : DNA

<211> Length : 40

SequenceName : Sequenz 9

25

SequenceDescription :

Feature

-----

Sequence: Sequenz 9:

30

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 40

Other Information :

CDSJoin : No

35

Custom Codon

-----

Sequence Name : Sequenz 9

40

Sequence

-----

<213> OrganismName : Artificial Sequence

5 <400> PreSequenceString :  
cagaatcagt aagattatca tcatagtctt ccgcttcttc 40  
<212> Type : DNA  
<211> Length : 40  
SequenceName : Sequenz 10  
10 SequenceDescription :

Feature  
-----

Sequence: Sequenz 10:

15 <221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
<222> LocationTo : 40  
Other Information :  
CDSJoin : No

20 Custom Codon  
-----

Sequence Name : Sequenz 10

25 Sequence  
-----  
<213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
ggtcaggttt gatgatgacg actctccttc ctttatcc 38

30 <212> Type : DNA  
<211> Length : 38  
SequenceName : Sequenz 11  
SequenceDescription :

35 Feature  
-----

Sequence: Sequenz 11:

<221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
40 <222> LocationTo : 38  
Other Information :  
CDSJoin : No

5

Custom Codon

-----

Sequence Name : Sequenz 11

10

Sequence

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

ggataaagga aggagagtcg tcatcatcaa acctgacc

38

15

<212> Type : DNA

<211> Length : 38

SequenceName : Sequenz 12

SequenceDescription :

20

Feature

-----

Sequence: Sequenz 12:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

25

<222> LocationTo : 38

Other Information :

CDSJoin : No

Custom Codon

30

-----

Sequence Name : Sequenz 12

Sequence

-----

35

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

cccttagtcc tcgccccctc tgacagaagt tataaaag

38

<212> Type : DNA

<211> Length : 38

40

SequenceName : Sequenz 13

SequenceDescription :

5 Feature

-----

Sequence: Sequenz 13:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

10 <222> LocationTo : 38

Other Information :

CDSJoin : No

Custom Codon

15 -----

Sequence Name : Sequenz 13

Sequence

-----

20 <213> OrganismName : Artificial Sequence

<400> PreSequenceString :

cttttataac ttctgtcaga gggggcgagg actaaggg

38

<212> Type : DNA

<211> Length : 38

25 SequenceName : Sequenz 14

SequenceDescription :

Feature

-----

30 Sequence: Sequenz 14:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 38

Other Information :

35 CDSJoin : No

Custom Codon

-----

Sequence Name : Sequenz 14

40

Sequence

-----

5 <213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
gtccgattta tggcatacac agatgttacc ttttaagactc g  
<212> Type : DNA  
<211> Length : 41  
10 SequenceName : Sequenz 15  
SequenceDescription :

Feature

-----

15 Sequence: Sequenz 15:  
<221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
<222> LocationTo : 41  
Other Information :  
20 CDSJoin : No

Custom Codon

-----

Sequence Name : Sequenz 15

25

Sequence

-----

<213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
30 cgagtcttaa aggtaacatc tgtgtatgcc ataaatcgga c  
<212> Type : DNA  
<211> Length : 41  
SequenceName : Sequenz 16  
SequenceDescription :

35

Feature

-----

Sequence: Sequenz 16:  
<221> FeatureKey : primer\_bind  
40 <222> LocationFrom : 1  
<222> LocationTo : 41  
Other Information :

5 CDSJoin : No

Custom Codon

-----

Sequence Name : Sequenz 16

10

Sequence

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

15

cctttaagac tcgtaaagct attcagcatg aatcagg

37

<212> Type : DNA

<211> Length : 37

SequenceName : Sequenz 17

SequenceDescription :

20

Feature

-----

Sequence: Sequenz 17:

<221> FeatureKey : primer\_bind

25

<222> LocationFrom : 1

<222> LocationTo : 37

Other Information :

CDSJoin : No

30

Custom Codon

-----

Sequence Name : Sequenz 17

Sequence

35

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

cccgattcat gctgaatagc tttacgagtc ttaaagg

37

<212> Type : DNA

40

<211> Length : 37

SequenceName : Sequenz 18

SequenceDescription :

5

Feature

-----

Sequence: Sequenz 18:

<221> FeatureKey : primer\_bind

10

<222> LocationFrom : 1

<222> LocationTo : 37

Other Information :

CDSJoin : No

15

Custom Codon

-----

Sequence Name : Sequenz 18

Sequence

20

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

cacactgttg attatattta agaataaago aagcagacca tataac

46

<212> Type : DNA

25

<211> Length : 46

SequenceName : Sequenz 19

SequenceDescription :

Feature

30

-----

Sequence: Sequenz 19:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 46

35

Other Information :

CDSJoin : No

Custom Codon

-----

40

Sequence Name : Sequenz 19

Sequence

5

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

gttatatggt ctgcttgctt tattcttaaa tataatcaac agtgtg

46

<212> Type : DNA

10

<211> Length : 46

SequenceName : Sequenz 20

SequenceDescription :

Feature

15

-----

Sequence: Sequenz 20:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 46

20

Other Information :

CDSJoin : No

Custom Codon

-----

25

Sequence Name : Sequenz 20

Sequence

-----

<213> OrganismName : Artificial Sequence

30

<400> PreSequenceString :

ccctcacgga atcactgatg tctctccttt gtattcaagg

40

<212> Type : DNA

<211> Length : 40

SequenceName : Sequenz 21

35

SequenceDescription :

Feature

-----

Sequence: Sequenz 21:

40

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 40



5        Other Information :  
         CDSJoin : No

         Custom Codon  
         -----

10      Sequence Name : Sequenz 21

         Sequence  
         -----

         <213> OrganismName : Artificial Sequence

15      <400> PreSequenceString :

         ccttgaatac aaaggagaga catcagtgat tccgtgaggg

40

         <212> Type : DNA

         <211> Length : 40

         SequenceName : Sequenz 22

20      SequenceDescription :

Feature  
-----

Sequence: Sequenz 22:

25      <221> FeatureKey : primer\_bind

         <222> LocationFrom : 1

         <222> LocationTo : 40

         Other Information :

         CDSJoin : No

30

         Custom Codon  
         -----

         Sequence Name : Sequenz 22

35      Sequence  
         -----

         <213> OrganismName : Artificial Sequence

         <400> PreSequenceString :

         gatgtccgtc ctttgtattc agggagatta ccaaaaagg

38

40      <212> Type : DNA

         <211> Length : 38

         SequenceName : Sequenz 23

5           SequenceDescription :

Feature

-----

Sequence: Sequenz 23:

10   <221> FeatureKey : primer\_bind

<222> LocationFrom : 1

<222> LocationTo : 38

Other Information :

CDSJoin : No

15

Custom Codon

-----

Sequence Name : Sequenz 23

20   Sequence

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

ccttttggtga atctccctga atacaaagga cggacatc

38

25   <212> Type : DNA

<211> Length : 38

SequenceName : Sequenz 24

SequenceDescription :

30   Feature

-----

Sequence: Sequenz 24:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

35   <222> LocationTo : 38

Other Information :

CDSJoin : No

Custom Codon

-----

40

Sequence Name : Sequenz 24

5 Sequence

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

ctgtatttga tgagaaccaa agctggtacc tcacag

10 <212> Type : DNA

<211> Length : 36

SequenceName : Sequenz 25

SequenceDescription :

15 Feature

-----

Sequence: Sequenz 25:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

20 <222> LocationTo : 36

Other Information :

CDSJoin : No

Custom Codon

25 -----

Sequence Name : Sequenz 25

Sequence

-----

30 <213> OrganismName : Artificial Sequence

<400> PreSequenceString :

ctgtgaggta ccagotttgg ttctcatcaa atacag

<212> Type : DNA

<211> Length : 36

35 SequenceName : Sequenz 26

SequenceDescription :

Feature

-----

40 Sequence: Sequenz 26:

<221> FeatureKey : primer\_bind

<222> LocationFrom : 1

36

36

5 <222> LocationTo : 36  
Other Information :  
CDSJoin : No

Custom Codon  
-----

10 Sequence Name : Sequenz 26

Sequence  
-----

15 <213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
ctccccaatc cagatggagt gcagcttgag  
<212> Type : DNA  
<211> Length : 30

20 SequenceName : Sequenz 27  
SequenceDescription :

Feature  
-----

25 Sequence: Sequenz 27:  
<221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
<222> LocationTo : 30  
Other Information :  
30 CDSJoin : No

Custom Codon  
-----

Sequence Name : Sequenz 27

35 Sequence  
-----

<213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
40 ctcaagctgc actccatctg gattggggag  
<212> Type : DNA  
<211> Length : 30

30

30

5       SequenceName : Sequenz 28  
      SequenceDescription :

Feature  
-----

10      Sequence: Sequenz 28:  
      <221> FeatureKey : primer\_bind  
      <222> LocationFrom : 1  
      <222> LocationTo : 30  
      Other Information :  
15       CDSJoin : No

Custom Codon  
-----

Sequence Name : Sequenz 28

20

Sequence  
-----

<213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
25      cagctggagt gcagcttcag gatccagagt tc  
      <212> Type : DNA  
      <211> Length : 32  
      SequenceName : Sequenz 29  
      SequenceDescription :

32

30

Feature  
-----

Sequence: Sequenz 29:  
35      <221> FeatureKey : primer\_bind  
      <222> LocationFrom : 1  
      <222> LocationTo : 32  
      Other Information :  
      CDSJoin : No

40

Custom Codon  
-----

Sequence Name : Sequenz 29

5

Sequence

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

10 gaactctgga tcttgaagct gcactccagc tg

32

<212> Type : DNA

<211> Length : 32

SequenceName : Sequenz 30

SequenceDescription :

15

Feature

-----

Sequence: Sequenz 30:

<221> FeatureKey : primer\_bind

20 <222> LocationFrom : 1

<222> LocationTo : 32

Other Information :

CDSJoin : No

25

Custom Codon

-----

Sequence Name : Sequenz 30

Sequence

30

-----

<213> OrganismName : Artificial Sequence

<400> PreSequenceString :

cgatggatc tgctcagcat gaagagcaat gaaaacatcc attctattc

49

<212> Type : DNA

35 <211> Length : 49

SequenceName : Sequenz 31

SequenceDescription :

Feature

40

-----

Sequence: Sequenz 31:

<221> FeatureKey : primer\_bind

5 <222> LocationFrom : 1  
<222> LocationTo : 49  
Other Information :  
CDSJoin : No

10 Custom Codon  
-----  
Sequence Name : Sequenz 31

Sequence  
15 -----  
<213> OrganismName : Artificial Sequence  
<400> PreSequenceString :  
gaatagaatg gatgttttca ttgctcttca tgctgagcag ataccatcg  
<212> Type : DNA  
20 <211> Length : 49

SequenceName : Sequenz 32  
SequenceDescription :

Feature  
25 -----  
Sequence: Sequenz 32:  
<221> FeatureKey : primer\_bind  
<222> LocationFrom : 1  
<222> LocationTo : 49  
30 Other Information :  
CDSJoin : No

Custom Codon  
-----  
35 Sequence Name : Sequenz 32

Sequence  
-----  
<213> OrganismName : Porcine  
40 <400> PreSequenceString :  
AIRRYILGAV ELSWDYRQSE LLRELHVDTR FPATAPGALP LGPSVLYKKT VFVEFTDQLF

5	SVARPRPPWM GLLGPTIQAE VYDTVVVTLK NMASHPVSLH AVGVSWFKSS EGAEYEDHTS	120
	QREKEDDKVL PGKSQTYVWQ VLKENGPTAS DPPCLTYSYL SHVDLVKDLN SGLIGALLVC	180
	REGSLTRERT QNLHEFVLLF AVFDEGKSWH SARNDSWTRA MDPAPARAQP AMHTVNGYVN	240
10	RSLPGLIGCH KKSVMWHVIG MGTSPPEVHSI FLEGHTFLVR HHRQASLEIS PLTFLTAQTF	300
	LMDLGQFLLF CHISSHHHGG MEAHVRVESC AEERQLRRKA DEEDYDDNL YDSMDVVRL	360
15	DGDDVSPFIQ IRSVAKKHPK TWHYISAEE EDWDYAPAVP SPSDRSYKSL YLNSGPQRIG	420
	RKYKKARFVA YTDVTFKTRK AIPYESGILG PLYGEVGDY LLIIFKNKAS RPYNIYPHGI	480
	TDVSALHPGR LLKGWKHLKD MPILPGETFK YKWTVTVEDG PTKSDPRCLT RYYSSSINLE	540
20	KDLASGLIGP LLICYKESVD QRGNQMMSDK RNVHDFSVFD ENQSWYLAEN IQRFLPNPDG	600
	LQPQDPEFQA SNIMHSINGY VFDSLQLSVC LHEVAYWYIL SVGAQTDFLS VFESQYTFKH	660
25	KMVEDTLTL FPFSGETVFM SMENPGLWVL GCHNSDLRNR GMTALLKVYS CDRDIGDYD	720
	NTYEDIPGFL LSGKNVIEPR SFAQNSRPPS ASQKQFQIT SPEDDVLDLP QSGERTQALE	780
	ELSVPSGDGS MLLGQNPAPH GSSSSDLQEA RNEADDYLPG ARERNTAPSA AARLRPELHH	840
30	SAERVLTPEP EKELKKLDSK MSSSSDLLKT SPTIPEDTLS AETERTHSLG PPHRQVNFRS	900
	QLGAIVLGKN SSHFIGAGVP LGSTEEDHES SLGENVSPVE SDGIFEKERA HGPASLTAKD	960
35	VLFKVNISLV KTKARVYLK TNRKIHIDDA ALLTENRASA TFMDKNNTAS GLNHVSNWIK	1020
	GPLGKNPLSS ERGPSPELLT SSGSGKSVKG QSSGQGRIRV AVEEEELSKG KEMMLPNSEL	1080
	TFLTNSADVQ GNDTHSQGKK SREEMERREK LVQEKVDLPQ VYTATGTKNF LRNIFHQSTE	1140
40	PSVEGFDGGS HAPVPQDSRS LNSAERAET HIAHFAIRE EAPLEAPGNR TGPGRSAVP	1200



5 RRVKQSLKQI RLPLEEIKPE RGVVLNATST RWESESPILQ GAKRNNLSLP FLTLEMAGGQ 1260  
GKISALGKSA AGPLASGKLE KAVLSSAGLS EASGKAEFLP KVRVHREDLL PQKTSNVSCA 1320  
HGD LGQEIFL QKTRGPVNLN KVNRPGRTPS KLLGFPMPKE WESLEKSPKS TALRTKDIIS 1380  
10 LPLDRHESNH SIAAKNEGQA ETQREAAWK QGGPGRLCAP KPPVLR RHQR DISLPTFQPE 1440  
EDKMDYDDIF STETKGEDPD IYGEDENQDP RSFQKRTRHY FIAAVEQLWD YGMSESPRAL 1500  
15 RNRAQNGEVP RFKKVVFREF ADGSFTQPSY RGELNKH LGL LCPYIRAEVE DNIMVTFKNQ 1560  
ASRPYSFYSS LISYPDDQEQ GAEPRHNFVQ PNETRTYFWK VOHHMAPTED EFDCKAWAYF 1620  
SDVDLEKDVH SGLIGPLLIC RANTLNAAHG RQVTYQEFAL FFTIFDETKS WYFTENVERN 1680  
20 CRAPCHLOME DPTLKENYRE HAINGYVMDT LPGLVMAQNO RIRWYLLSMG SNENIHSIHF 1740  
SGHVFSVRKK BEYKMAVYNL YPGVFETVEM LPSKVGIWRI ECLIGEHLQA GMSTTFLVYS 1800  
25 KECQAPLGMA SGRIRDFQIT ASGOYGQWAP KLARLHYS GS INAWSTKDPH SWIKVDLLAP 1860  
MIIHGIMTQG ARQKFSSLYI SQFIIMYSLD GRNWQSYRGN STGTLMVFFG NVDASGIKHN 1920  
IFNPPIVARY IRLHPTHYSI RSTLRMELMG CDLNSCSMPL GMQNK AISDS QITASSHLSN 1980  
30 IFATWSPSQA RLHLQGR TNA WRPRVSSAEE WLQVDLQKTV KVTGITTQGV KSLLSSMYVK 2040  
EFLVSSSQDG RRWTLFLQDG HTKVFQGNQD SSTPVVNALD PPLFTRYLRI HPTSWAQHIA 2100  
35 LRLEVLGCEA QDLY 2114  
<212> Type : PRT  
<211> Length : 2114  
SequenceName : Sequenz 33  
SequenceDescription :

40

Feature

-----

5 Sequence: Sequenz 33:  
<221> FeatureKey : CHAIN  
<222> LocationFrom : 1  
<222> LocationTo : 2114  
Other Information :  
10 CDSJoin : No

Sequence  
-----

15 <213> OrganismName : human  
<400> PreSequenceString :  
ATTRYYLGAV ELSWDYMQSD LGELPVDARF PPRVPKSPFF NTSVVYKCTL FVEFTDHLFN 60  
IAKPRPPWMG LLGPTIQAEV YDTVVITLKN MASHFVSLHA VGVSYWKASE GAHYDDQTSQ 120  
20 REKEDDKVFP GGSHTYVWQV LKENGPMASD PLCLTYSYLS HVDLVKDLNS GLIGALLVCR 180  
EGSLAKEKTO TLHKFILLFA VFDEGKSWHS ETXNSLMQDR DAASARAWPK MHTVNGYVNR 240  
SLPGLIGCHR KSVYWHVIGM GTTPEVHSIF LEGHTFLVRN HRQASLEISP ITFLTAOTLL 300  
25 MDLGQFLLFC HISSHQHDGM EAYVKVDSCP EEPQLRMKNN EEAEDYDDDL TDSEMDVVRP 360  
DDDNPSFSIQ IRSVAKKHPK TWVHYIAAEE EDWDYAPLVL APDDRSYKSQ YLNNGPQRIG 420  
30 RKYKKVRFMA YTDFTFKTRE AIQHESGILG PLYGEVGDY LLIIFKNQAS RPYNIYPHGI 480  
TDVRPLYSRR LPKGVKHLKD FPILPGEIFK YKWTVTVEDG PTKSDPRCLT RYSSSFVNME 540  
RDLASGLIGP LLICYKESVD QRGNQIMSDK RNVILFSVFD ENRSWYLTEN IQRFDPNPAG 600  
35 VQLEDPEFQA SNIMHSINGY VFDSLQLSVC LHEVAYWYIL SIGAQTDFLS VFFSGYTFFH 660  
KMVYEDTLTL FPFSGETVFM SMENPGLWIL GCHNSDFRNR GMTALLKVSS CDKNTGDYEE 720  
40 DSYEDISAYL LSKNNAIEPR SFSQNSRHRS TRQKQFNATT IPENDIEKTD PWFHRTMPM 780

5 KIQNVSSSDL LMLLRQSPTP HGLSLSDLQE AKYETFSDDP SPGAIDSNNNS LSEMTHFRPQ 840

LHHSGDMVFT PESGLQLRLN EKLGTAAATE LKKLDFKVSS TSNNLISTIP SDNLAAGTDN 900

10 TSSLGPPSMP VHYDSQLDTT LFGKKSSPLT ESGGPLSLSE ENNDSKLLES GLMNSQESSW 960

GKNVSSTESG RLFKGKRAHG PALLTKDNAL FKVSISLLKT NKTSNNSATN RKTHIDGPSL 1020

LIENSPSVWQ NILESDETEFK KVTPLIHDRM LMDKNATALR LNHMSNKTTS SKNMEMVQOK 1080

15 KEGPIPPDAQ NPDMSFFKML FLPE SARWIQ RTHGKNSLNS GQGPSPKQLV SLGPEKSVEG 1140

QNFLSEKNKV VVGKGFTKD VGLKEMVFP SRNLFTNLD NLHENNTHNQ EKKIQEEIEK 1200

KETLIQENVV LPQIHTVTGT KNFMKNLFL STONVEGYSY DGAYAPVLQD FRSNDSTNR 1260

20 TKKHTAHFSK KGEENLEGL GNQTKQIVEK YACTTRISPN TSQONFVTOR SKRALKQFRL 1320

~~PLEETELEKR IIVDDTSTQW SKNMKHLTPS TLTOIDYNEK EKGAITQSPL SDCLTRSHSI 1380~~

25 PQANRSPLPI AKVSSFPSIR PIYLTRYLFQ DNSSHLPAAS XRKKDSGVQE SSHFLQGAKK 1440

NNLSLAILTL EMTGDQREVG SLGTSATNSV TYKKVENTVL PKPDLPKTSK KVELLPKVHI 1500

30 YQKDLFPTET SNGSPGHLDL VEGSLLQGE GAIKWNEANR PGKVPFLRVA TESSAKTPSK 1560

LLDPLAWDNH YGTQIPKEEW KSQEKSPKT AFKKKDTILS LNACESNHAI AAINEGQNKP 1620

EIEVTWAKQG RTERLCSQNP PVLKRHQREI TRTTLOSDQE EIDYDDTISV EMKKEDFDIY 1680

35 DEDENQSPRS FQKKTRHYFI AAVERLWDYG MSSSPHVLRN RAQSGSVQOF KKVVFQEFDT 1740

GSFTQPLYRG ELNEHLGLLG PYIRAEVEDN IMVTFRNQAS RPYSFYSSLI SYEEDQRQGA 1800

EPRKQYFKPN ETKTYFWKVQ HHMAPTKDEF DCKAWAYFSD VDLEKDVHSG LIGPLLVCHT 1860

40 NTLNPAHGRQ VTVQEFALFF TIFDETKSWY FTE NMERNCR APCNIQMEDP TFKENYRFHA 1920

5 INGYIMDTLP GLVMAQDQRI RWYLLSMGSN ENIHSIHFSG HVFTVRKKEE YKMALYNLYP 1980  
GVFETVEMLP SKAGIWRVEC LIGEHLHAGM STLFVYSNK CQTPLG MASG HIRDFQITAS 2040  
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5     Aventis Behring GmbH

2002/M018 (A66)

Dr. Pfe/vm

10    **Claims:**

15    1.     Modified human factor VIII cDNA wherein mutations are inserted either in the wild-type factor VIII cDNA or in a factor VIII cDNA in which the B-domain is partially or completely deleted and may be replaced by a DNA linker segment, characterised in that

20       A)    one or several codons of the human factor VIII cDNA which are not identical with the corresponding codon in the same position of the porcine factor VIII cDNA are substituted by a different codon in such a way that

25       •     when the human sequence contains a codon for a neutral amino acid whereas the porcine sequence contains a codon for a charged amino acid then a codon for an amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

30       •     when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for a neutral amino acid then a codon for a neutral amino acid or a codon for an amino acid of the opposite charge is introduced into the human sequence,

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- 5
- when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for an amino acid with the opposite charge then a codon for an amino acid with the opposite charge is introduced into the human sequence or

10 B) one or several codons for a charged amino acid which are found in the FVIII cDNA of a hemophilic patient are replaced by a codon for an amino acid of the opposite charge.

2. Recombinant expression vector containing the factor VIII cDNA as claimed in  
15 claim 1, **characterised in that** it carries in addition transcriptional regulatory elements for expression in a suitable host cell.

3. Modified biologically active recombinant human factor VIII with improved stability wherein mutations are inserted either in the wild-type factor VIII or in a  
20 factor VIII in which the B-domain is partially or completely deleted and may be replaced by a linker, **characterised in that**

A.) one or several amino acids of the human factor VIII which are not identical with the corresponding amino acid in the same position of the porcine factor  
25 VIII are substituted by a different amino acid in such a way that

• when the human sequence contains a neutral amino acid whereas the porcine sequence contains a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;  
30

• when the human sequence contains a charged amino acid whereas the porcine sequence contains a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge is introduced into the human sequence;  
35

5

- when the human sequence contains a charged amino acid whereas the porcine sequence contains an amino acid with the opposite charge then an amino acid with the opposite charge is introduced into the human sequence or

10

B) one or several charged amino acids which are found in the FVIII amino sequence of hemophilic patients are replaced by a codon for an amino acid of the opposite charge.

15

4. Modified biologically active recombinant human factor VIII as claimed in claim 3, wherein the plasma half life of its activated form is more than 3 minutes, preferably more than 10 minutes and most preferably more than 30 minutes.

20

5. Modified human factor VIII, **characterised in that** its A2-domain is stabilised by the substitution of one or several amino acids as claimed in claim 3.

25

6. Process for the recombinant production of a modified human factor VIII as claimed in claim 3 either in cell suspension or on a solid support as a batch cell culture or as a perfusion cell culture with continuous production of a conditioned medium **characterised in that** the factor VIII proteins, which are expressed by a suitable host cell line are purified by chromatographic methods.

30

7. Process as claimed in claim 6, **characterised in that** the transcription units encoding the modified factor VIII cDNA of claims 1 and 2 contain a dominant selectable marker in order to facilitate the isolation of specific cell clones which have integrated said specific c-DNA into their genome.

35

8. Host cell line for expression of the factor VIII proteins of claim 3, **characterised in that** it is an animal cell line of vertebrate origin which contains the factor VIII cDNA of claim 1 integrated into its genome.

5

9. Pharmaceutical composition, characterised in that it comprises a modified biologically active recombinant human factor VIII of claim 3.

10. Vector for gene therapy of hemophilia A, characterized in that it contains a modified FVIII cDNA as claimed in claim 1.



5     Aventis Behring GmbH

2002/M018 (A66)

Dr. Pfe/vm

10    Abstract:

**Modified cDNA Factor VIII and its Derivatives**

15    Modified human factor VIII cDNA wherein mutations are inserted either in the wild-type factor VIII cDNA or in a factor VIII cDNA in which the B-domain is partially or completely deleted and may be replaced by a DNA linker segment and

20           A) one or several codons of the human factor VIII cDNA which are not identical with the corresponding codon in the same position of the porcine factor VIII cDNA are substituted by a different codon in such a way that

25           •     when the human sequence contains a codon for a neutral amino acid whereas the porcine sequence contains a codon for a charged amino acid then a codon for an amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

30           •     when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for a neutral amino acid then a codon for a neutral amino acid or a codon for an amino acid of the opposite charge is introduced into the human sequence,

35           •     when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for an amino

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acid with the opposite charge then a codon for an amino acid with the opposite charge is introduced into the human sequence or

10

B) one or several codons for a charged amino acid which are found in the FVIII cDNA of a hemophilic patient are replaced by a codon for an amino acid of the opposite charge.